

## **Evolutionary Significance of Seed Structure in Alpinioideae (Zingiberaceae)**

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## ABSTRACT

The Alpinioideae are the largest of the four subfamilies in Zingiberaceae and are widely distributed throughout the new and old world tropics. Recent molecular studies have shown that although Alpinioideae are a strongly supported monophyletic subfamily with two distinct tribes (Alpinieae and Riedelieae), large genera such as *Alpinia* and *Amomum* are polyphyletic and are in need of revision. *Alpinia* has been shown to form seven distinct clades and *Amomum* three, but for many of these clades, traditional vegetative and floral synapomorphies have not been found. A broad survey of seeds within Alpinioideae using light microscopy and synchrotron based X-ray tomographic microscopy shows that many clades have distinctive seed structures that serve as distinctive apomorphies. Tribes Riedelieae and Alpinieae can be distinguished based on operculum structure with the exception of three taxa analyzed. The most significant seed characters were found to be various modifications of the micropylar and chalazal ends, the cell shape of the endotesta and exotesta, and the location of an endotestal gap. A chalazal chamber and hilar rim are reported for the first time in Zingiberaceae. In addition to characterizing clades of extant lineages, these data offer insights into the taxonomic placement of many fossil zingiberalean seeds that are critical to understanding the origin and evolution of Alpinioideae and Zingiberales as a whole.

Keywords: chalaza; chalazal chamber; embryo; mesotesta; micropyle; operculum; seed; *Spirematospermum*; synchrotron based X-ray tomographic microscopy (SRXTM); testa.



## INTRODUCTION

The ginger family, Zingiberaceae Martinov, is the largest of eight families in Zingiberales Griseb. and is distributed throughout the old and new world tropics with a center of diversity in Asia (Larsen *et al.*, 1998; Larsen, 2005). The members of the family are easily differentiated from other Zingiberales by a distinct labellum with two fused adaxial staminodes, two nectariferous glands at the base of the style, and perhaps most notably by the presence of ethereal oils found throughout the vegetative organs of the plant, which give gingers their unique flavor and smell (Kress, 1990; Larsen *et al.*, 1998; Kress *et al.*, 2001; Pedersen, 2003). Traditionally, the family was divided into four tribes based on a suite of morphological characters; however, the characters were not uniquely distributed within a single tribe or were not present in all members of the tribe (Kress, Prince & Williams, 2002; Pedersen, 2003). To address this issue, Kress *et al.* (2002) analyzed plastid *matK* and nuclear rDNA ITS sequences and revised the family to include four well-supported subfamilies; two early diverging subfamilies Siphonochiloideae Kress (2 genera/ 17 species) and Tamijioideae Kress (1 genus/ 1 species), and two large subfamilies Alpinioideae Link (20 genera/ ~900 species) and Zingiberoideae Haask. (30 genera/ ~680 species) (Harris *et al.*, 2006; The Plant List, 2013).

Alpinioideae are the largest subfamily within Zingiberaceae and are characterized by having two very reduced or absent lateral staminodes and a plane of distichy perpendicular to the growth of the rhizome (Burt, 1972; Kress *et al.*, 2002). The two recognized tribes within Alpinioideae, Riedelieae Kress and Alpinieae A. Rich., can be easily distinguished from each other as Riedelieae members possess long silique-like

capsules that dehisce basally and extrafloral nectaries on the midrib of the adaxial surface of the leaf blade (Kress *et al.*, 2002). In contrast, members of the Alpinieae often possess globose or ovoid fruits that are not as elongated as those seen in Riedelieae, and lack extrafloral nectaries (Smith, 1972, 1990b; Larsen & Mood, 1998; Kress *et al.*, 2002). Although molecular and morphological data strongly support the monophyly of these two tribes, intergeneric relationships within Alpinieae are not well-supported and recent studies have shown instances of paraphyly and polyphyly throughout the tribe, most notably in *Alpinia* Roxb. and *Amomum* Roxb. (Rangsiruji, Newman & Cronk, 2000a, 2000b; Xia, Kress & Prince, 2004; Kress *et al.*, 2005, 2007; Kaewrsi *et al.*, 2007).

A formal revision to the Alpinioideae has yet to be published to address these incongruences in phylogeny, but well-supported informal clades based on ITS and *trnK/matK* sequence data have been proposed and include seven distinct clades of *Alpinia* taxa (*Alpinia carolinensis* clade, *Alpinia eubractea* clade, *Alpinia fax* clade, *Alpinia galanga* clade, *Alpinia rafflesiana* clade, *Alpinia zerumbet* clade, and *Alpinia zerumbet* subclade) and three clades of *Amomum* (*Amomum maximum* clade, *Amomum tsao-ko* clade, and *Amomum villosum* clade; Kress *et al.*, 2005, 2007). The proposed clades have some similarity to previous classification schemes based on morphological data (Schumann, 1904; Holttum, 1950; Wu, 1981; Smith, 1990a), but the majority are newly recognized relationships with few apomorphies due in part to the fact that many morphological characters used to reconstruct phylogenies or define lineages have been shown to be homoplasious [e.g., presence of bracteoles by Schumann (1904), and labellum shape by Smith (1990)].

Currently, the most well-known and commonly used morphological characters for taxon identification and phylogenetic reconstruction are those from flowers and inflorescences; however seed morphology and anatomy are also excellent sources of potentially phylogenetically informative characters (Liao & Wu, 1996, 2000; Tang *et al.*, 2005) and seeds are more readily preserved as fossils than flowers (e.g., Koch & Friedrich, 1971; Friis, 1988; Manchester & Kress, 1993; Rodriguez-de la Rosa & Cevallos-Ferriz, 1994; Fischer *et al.*, 2009). Several taxonomically useful characters from fruits and seeds have been previously documented for Zingiberales (Gootjen & Bouman, 1981; Manchester & Kress, 1993; Rodriguez-de la Rosa & Cevallos-Ferriz, 1994; Liao & Wu, 1996, 2000; Liao *et al.*, 2004; Tang *et al.*, 2005; Benedict, 2012). Within seeds, however, characters derived from the aril, operculum, micropylar collar, perisperm, and endosperm, coupled with characters from seed coat anatomy, embryo shape, and ovule type, have all been underutilized in understanding phylogenetic relationships and character evolution in the group (Kress *et al.*, 2001, 2002, 2005, 2007). Such characters would not only be useful for elucidating relationships and defining potential synapomorphies for extant lineages, but would facilitate the incorporation of fossil taxa into phylogenetic analyses.

The first studies of seed and fruit characters in the Zingiberales can be traced back to Tschirch (1891), Humphry (1896), and Netolitzky (1926) and Mauritzon (1936), which are summarized along with other work by Takhtajan (1985) to give general descriptions of the seed and fruit characters at the family level. Takhtajan (1985) noted that fruits of Zingiberaceae tend to be many seeded, are sometimes fleshy, and can be loculicidally, septicidally or irregularly dehiscent. Seeds are most often anatropous, although

campylotropous ovules were noted in *Hedychium* J. Koenig. (Zingiberoideae) and orthotropic ovules in *Cucurma caulina* J. Graham (Zingiberoideae, Takhtajan, 1985). Seed coats in Zingiberaceae, as well as many other taxa in Zingiberales, are formed from the outer integument only (Takhtajan, 1985), and it is here that considerable anatomical variation exists. Takhtajan (1985) noted a range of 5-13 layers of cells that comprise the seed coat, but did not comment on any characters that may be useful for unifying clades of the family. The variation of seed morphology and anatomy within Zingiberaceae has not been well-studied, and the only research conducted in a systematic context is that of Liao and Wu (2000), who demonstrated differences of seed anatomy between the two large subfamilies Alpinioideae and Zingiberoideae.

The markedly revised phylogenetic hypothesis of Alpinioideae (Kress *et al.*, 2005, 2007) coupled with a broad sampling of taxa within the group allows for a reinvestigation of seed characters in a systematic context. The aim of this study is three fold: 1) to revise and standardize terminology previously used for Zingiberaceae seeds, 2) to assess if there are additional variable characters that have not been previously and 3) to analyze seed characters in the context of the recently proposed phylogenetic hypothesis of Alpinioideae (Kress *et al.*, 2007) to determine if synapomorphies can be defined for the newly proposed clades.

## MATERIALS & METHODS

Forty-two species belonging to 15 genera of Alpinioideae were sampled from various herbaria, botanical gardens, or purchased for use in this study (Table 1). The number of seeds available for analysis varied from one to more than 50 for each taxon.

Specimens were either thin sectioned and observed with light microscopy, or analyzed using synchrotron based X-ray tomographic microscopy (SRXTM; also known as synchrotron radiation X-ray computed tomography, SRXCT). All seeds of Zingiberaceae have arils and they were not physically removed from seeds that were analyzed, but in rare cases the aril fell off a seed during preparation for analysis.

### **Thin sectioning and light microscopy**

Seeds of Zingiberales contain silica cells (phytoliths) that can make traditional microtomy impractical without the use of hydrofluoric acid to first dissolve the siliceous bodies (Liao & Wu, 1996; 2000). Therefore material was embedded in Ward's Bio-plastic Synthetic Resin following the manufacture's protocol (Ward's Natural Science, Rochester, NY) and sectioned using a common protocol employed for fossilized material (Hass & Rowe, 1990; Benedict, Pigg & DeVore, 2008). Embedded seeds were longitudinally sectioned into wafers less than 1.0 mm thick on a Buhler Isomet low-speed lapidary saw or Buhler Isomet 1000 precision lapidary saw with a diamond blade (BUEHLER, a division of Illinois Tool Works Inc., Lake Bluff, Illinois, USA). Wafers were mounted on standard microscope slides using U-154 adhesive (The Company, Lakewood, Colorado, USA) and ground down to a minimal thickness using various grades of carborundum powder or sand paper until a single layer of cells was present on the slide. Specimens were photographed using a Nikon D70s or D90 camera body (Nikon Inc. Melville, NY, USA) attached to a Nikon SMZ 1500 stereoscope, a Nikon Eclipse E800 compound scope, or a Leica DM EP compound scope with dedicated Leica DFC290 camera attachment. Images were adjusted uniformly for contrast and color balance, and artifacts (bubbles, lint, etc.) were removed from the background only using

Adobe Photoshop software version CS or CS2 (Adobe Systems Incorporated San Jose, CA, USA).

### **Synchrotron based X-ray tomographic microscopy**

Samples were mounted onto brass stubs or toothpicks using a PVA glue or epoxy and imaged using standard absorption contrast at the TOMCAT beamline at the Swiss Light Source (SLS; Stampanoni *et al.*, 2006; Paul Scherrer Institut, Villigen, Switzerland; specimens scanned during sessions in 2009, 2010, 2011, and 2013); the 2-BM beamline at the Advanced Photon Source (APS; Argonne National Laboratory, Lemont, IL; specimens scanned during sessions in 2011 and 2012); or the 8.3.2 beamline at the Advanced Light Source (ALS; MacDowell *et al.*, 2012; Lawrence Berkeley National Laboratory, Berkeley, California; specimens scanned during session in 2013). Specimens were scanned as follows: At TOMCAT, transmitted X-rays were converted into visible light using a LAG:Ce 200  $\mu\text{m}$  scintillator (2009–2011), or a 20  $\mu\text{m}$  or 100  $\mu\text{m}$  LAG:Ce scintillator (2013) screen (Crytur, Turnov, Czech Republic). Projection data were magnified by 2x or 4x microscope objectives and digitized by a high-resolution CCD camera (PCO.2000; PCO GmbH, Kelheim, Germany; 2009–2011) or sCMOS camera (PCO.edge 5.5; PCO GmbH, Kelheim, Germany; 2013). Samples were scanned using 10 or 13 keV and an exposure time per projection of 50, 125, 150 or 200 milliseconds. For each scan, a total of 1501 projections (2048x2048 pixels with PCO.2000 camera, 2560x2160 pixels with PCO.edge 5.5 camera) were acquired over 180°. Reconstruction of the tomographic data were performed on a 60-node Linux PC cluster using a highly optimized routine based on the Fourier transform method and a gridding procedure

(Marone, Münch, & Stampanoni 2010; Marone & Stampanoni, 2012), resulting in a theoretical pixel size of 3.7  $\mu\text{m}$  at 2x and 1.85  $\mu\text{m}$  at 4x (2009-2011) or 3.25  $\mu\text{m}$  at 2x and 1.625  $\mu\text{m}$  at 4x (2013) for reconstructed images.

At 2-BM, transmitted x-rays were converted to visible light using a 100  $\mu\text{m}$  thick Ce-doped LAG scintillator screen (Crytur, Turnov, Czech Republic). 2.5x, 4x, or 5x microscope objectives were used to magnify the projection data, and a Coolsnap K4 camera (Photometrics, Tucson, Arizona) was used to digitize the data. Samples were scanned at 16.1 or 21 keV with an exposure time of 280–300 ms. For each scan, a total of 1500 projections (2048x2048 pixels) were acquired over 180°. The tomographic reconstructions were conducted with a 64-node cluster at APS using a gridrec reconstruction algorithm (Dowd *et al.* 1999). Reconstructed images had a theoretical pixel size of 2.1  $\mu\text{m}$  at 2.5x, 1.7  $\mu\text{m}$  at 4x, and 1.5  $\mu\text{m}$  at 5x.

At the 8.3.2 beamline, transmitted x-rays were converted to visible light using a 0.5 mm LuAG scintillator (Crytur, Turnov, Czech Republic). Samples were magnified with either a 2x or 5x microscope objective and digitized using a sCMOS camera (PCO.edge; PCO GmbH, Kelheim, Germany). Samples were scanned at 15 keV and an exposure time of 90, 500, or 950 ms. For each scan, a total of 2049 projections (2560x2160 pixels) were acquired over 180°. Reconstruction was carried out using a custom ImageJ (Rasband, 1997–2014) plugin for image preprocessing and Octopus (Inside Matters, Aalst, Belgium) for tomographic reconstruction. Reconstructed images had a theoretical pixel size of 3.25  $\mu\text{m}$  at 2x and 1.3  $\mu\text{m}$  at 5x.

Reconstructed images were processed at the University of Michigan using Avizo 7.0 or 8.0 (FEI Visualization Science Group, Burlington, Massachusetts) for Windows 7.

Images were captured in Avizo 7.0 or 8.0 and edited uniformly for contrast using Adobe Photoshop CS2.

## RESULTS

### Variation in seed structure

Twenty-three characters were examined to describe variation in seed structure within Alpinioideae. All seeds examined were mature, arillate, operculate with a micropylar collar, but varied in possessing a hilar rim, micropylar and chalazal mesotestal proliferation of cells, a chalazal chamber and chalazal mucro. In Alpinioideae as with all Zingiberaceae, the seed coat is derived from the outer integument only (Grootjen & Bouman, 1981) and consists of three easily distinguishable layers, the exotesta, mesotesta and endotesta (Fig. 1). A variety of terms have been used to describe zingiberalean seeds based on histological sections and surface fractures, and are discussed below as the examined characters are introduced.

1. Seed surface—The surfaces of Alpinioideae seeds range from striate (Fig. 2A), striate and shiny (Fig. 2B), verrucose (surfaces with small bumps, Fig. 2C) or rugose (distinctly wrinkled surfaces, Fig. 2D).

2. Seed shape—The overall shape of an individual seed in Zingiberaceae is greatly influenced by the number of seeds per locule, and taxa with multiple seeds per locule tend to have many variously shaped seeds due to tighter packing in the fruit (Benedict, pers. obs.). This is particularly evident in the seeds closest to the micropylar and chalazal regions as they are often compressed during fruit and seed development. Accounting for this phenomenon, the following seed shapes were identified; ellipsoid (Figs. 2E-J), ovoid (Figs. 2K, L), or oblate and flattened at the poles of the seed (Figs. 2M-P).



3. Seed length—As with seed shape, seed length may also vary considerably between seeds within a single fruit, but a binary character of either “twice as long as wide” (e.g., Figs. 2E, F, Q, R) or “less than twice as long as wide” (e.g., Figs. G-P) was used to generalize seed length.

4. Seed body taper—Generally seeds were found to taper at the chalazal region or base (Figs. 2Q, R), at the micropylar region or apex (Figs. 2K, L), at both regions, or display no tapering at all.

5. External raphe groove—Zingiberales have anatropous ovules and development of the embryo, endosperm, and perisperm produces distinct external characteristics (Grootjen & Bouman, 1981). In *Costus*, it was shown that a substantial increase of nucellus tissue enveloped the seed coat in the chalazal region, which resulted in a sunken chalaza of the mature seed (Grootjen & Bouman, 1981). It is presumed that a similar developmental event has taken place with the raphe in the mesotesta of Alpinioideae seeds because the seed coat is often depressed (grooved) where the raphe is located. This is apparent along a single side of the seed in many taxa (Figs. 2H, J, L, R at arrow), and in few taxa, this groove extends to both sides of the seed (Figs. 2E, F at arrow).

6. External chalazal indentation—In some taxa, the outer surface of the chalazal region has a small circular indentation that may (Fig. 2J, L) or may not (Fig. 2H) be accompanied by the external raphe groove (character 5 above). It is unclear whether this chalazal indentation is homologous to the sunken chalaza of *Costus* L. (Grootjen & Bouman, 1981) and future work on the development of this trait is needed.

7. Operculum layering—An operculum is found in all taxa in Alpinioideae and located within the tubular micropylar collar. It is a more or less conical structure formed

from the inner layer(s) of the outer integument that seals the embryo cavity, and is pushed off by the embryo during germination (Figs. 3A-H, Figs. 4A-J). In all taxa observed the sclerotic endotesta contributes to the operculum, and in some taxa, a layer of mesotestal cells also form part of the operculum. In SRXTM images, the endotesta is often delimited by X-ray bright cells (appearing white; e.g., Figs. 3B, D at arrow, 4H-J) and the mesotesta is found above this layer as either large thin-walled parenchymatous cells (Figs. 3D, H) or a dense mass of cells (Fig. 3B).

8. Micropylar collar—The micropylar collar is a cylindrical expansion of mesotesta and endotesta into the embryonic chamber. It completely surrounds the operculum and often houses a portion of the embryo closest to the micropyle (Figs. 3A-H, 4A-J). It is present in all Alpinioideae studied.

9. Micropylar collar layering—As with the operculum, the micropylar collar is formed from either of endotestal cells only, or of both endotestal and mesotestal cells. Three types of micropylar collars have been documented previously in Zingiberaceae based on the relative abundance of mesotestal cells in the collar (Figures 2A-C in Liao & Wu, 1996). They range from those with large volume mesotestal cells (“form A”) to those with mesotestal cells of small volume (“form B”), to those with little or no mesotestal cells (“form C”). Only two micropylar collar forms were recognized in Alpinioideae, those that contain mesotesta (Figs. 3A, B, G, H, 4A-G) and those with no mesotesta (Figs. 3C-F). The distinction of large and small mesotestal cells (forms A and B) was found to be very subtle and could not be determined with confidence in this study.

10. Hilar rim—The hilar rim was first described in *Ensete* Horan. (Musaceae Juss.) as a rimmed hilar depression that in longitudinal section “produces the appearance of a

pair of horns arising from the hilar end of the seed” (Manchester & Kress, 1993:1267).

The definition of this character has been adopted with a slight modification to define it as a tubular outgrowth of exotestal cells at micropylar region of the seed (Figs. 4A-D, F).

11. Micropylar mesotestal proliferation—Another distinct feature of the micropylar region in some Alpinioideae is the proliferation of mesotestal cells in the micropylar region to produce a mass of cells in the shape of a three-dimensional torus, or doughnut. In longitudinal section this proliferation of cells can be seen situated between the hilar rim and micropylar collar (Figs. 4A, B, D).

12. Chalazal modification—Alpinioideae seeds range from having no cellular modifications in the chalazal region to various modifications that limit the amount of space available for the embryo and the embryo cavity (Figs. 5A-G). Chalazal modifications are divided into two general forms; chalazal chambers and testal proliferations. Chalazal chambers are empty cavities nested within the mesotesta of seed coats (Figs. 5C-F, at arrow; see character 13). Testal proliferations are masses of mesotestal cells that can fill up to almost half the volume of a mature seed (Figs. 5A-F). Testal proliferations do not include raphe and chalazal pigment group cells (see character 21). Two types of testal proliferations exist. One is a simple mass of mesotestal cells (Figs 5A-D, F). The other, termed here a columnar mesotestal proliferation, is a wall or column of endotestal and mesotestal cells that split the lower portion of the embryo cavity into two segments (Fig. 5E). These two chalazal modifications are not exclusive and taxa can have both a chamber and a proliferation of mesotestal cells (Figs. 5C-F).

13. Chalazal chamber size—We observed two types of chalazal chambers in Alpinioideae seeds: an *Alpinia*-type chamber that is smaller than 1/3 the width of the seed

(Fig. 3E), and an *Amomum*-type chamber that is defined as being 1/3 the width of the seed or larger (Figs. 5C, D, F). The *Alpinia*-type chamber is restricted to the chalazal end of the seed, similar to the chalazal chambers found in Musaceae and Costaceae. In contrast, the *Amomum*-type chamber connects to a raphe canal in the mesotesta that extends to the micropylar region of the seed (Fig. 5D). This has not previously been reported, and without developmental studies it is not clear whether these are homologous structures or not. We have chosen to be conservative here and treat them as two variations of chalazal chamber rather than introduce a new term without evidence of their homology.

14. Chalazal mucro—The first and only report of a chalazal mucro was by Ridley (1909), where it was termed a “terminal mucro” and was suggested to be a modification of *Burbridgea* Hook. f. seeds for wind and water dispersal. It is a chalazal outgrowth of the endotesta, mesotesta, and exotesta that produces a distinct mucro in some seeds (Figs. 2Q, R, 5H, I).

15. Palisade exotesta—The exotesta is the outermost single layer of cells of the seed coat (Figs. 6A-R). In longitudinal section, the exotesta ranges from large rectangular palisade cells, to square shaped cells (Figs. 6C-L), to a thin layer of compressed cells that often lack cellular contents (Figs. 6M-R).

16. Uniform exotesta—The exotestal layer of cells is either a uniform layer of cells or a disorganized layer of cells that have undergone irregular anticlinal divisions (e.g., Fig. 6H). The uniformity of the exotesta may also be interrupted by an inconsistent expansion of the exotesta cells, which results in a wavy appearance of the exotesta in transverse section (Fig. 6I).

17. Large mesotestal cells—The mesotesta often comprises three distinct layers of cells, which have been described as the hypodermis, the translucent cell layer, and the pigment cell layer (Liao & Wu, 1996, 2000). The hypodermis is a single layer of cells adjacent to the exotesta and consists of cells with brown pigments (Fig. 6A). The translucent layer is also a single layer of cells, but without any pigments (Fig. 6A at arrow). The pigment layer can range from 2-5 or more cells thick and is adjacent to the endotesta. It is sometimes absent or reduced to very small cells (Figs. 6G, O, Q), or consists of large cells that account for a large portion of the seed coat (Figs. 6A-F, J-M, R).

18. Endotestal cell shape—The endotesta is the innermost layer of the seed coat and is typically a single layer of sclerified cells often with lumen contents in the outermost zone that are X-ray bright in SRXTM images. These cells range in shape from square (Figs. 6A-C) to rectangular (Figs. 6D, F) and in some taxa they are elongated into a layer of endotestal palisade cells (Figs. 3O-Q).

19. Uniform endotesta—The endotesta is often a uniform layer of cells throughout the seed coat, but sometimes this layer has regions that are differentially elongated, causing an irregular endotestal layer. This layer has been shown to be irregular in the literature, (Chen *et al.*, 1989; Fig. 1.5 of Liao & Wu, 1994) but no taxa analyzed here have regions of endotesta that are differentially elongated.

20. Endotestal gap location—In the chalazal region of a seed, the endotesta often contains a small circular or ellipsoid hole devoid of sclerenchymatous cells that represents the point at which the raphe terminates in the seed (Figs. 5A-G). The

endotestal gap is either located at the center of the chalazal region (Figs. 5A-C, E, G) or is situated on the side of the seed (Figs. 5D, F).

21. Chalazal pigment group and raphe—The chalazal pigment group (CPG, Liao & Wu, 1996, 2000) is a collection of cells that is located above the endotestal gap and is adjacent to the raphe (Figs. 5A-G). The shape of the CPG has been shown to vary within Zingiberaceae and is discoid in Alpinioideae and “trumpet shaped” in Zingiberoideae (Liao & Wu, 2000). In SRXTM images, the raphe and CPG are very difficult to differentiate and are scored together.

22. Embryo shape—Four basic embryo shapes were observed in alpinoid seeds: straight and elongate (Fig. 7A), L-shaped (Figs. 7B-D), basally bulbous (Figs. 7E), or forked, with the base of the embryo splitting into two halves (Fig. 7F)

23. Embryo-endotesta contact—Embryo, perisperm, and endosperm fill the embryo cavities of Alpinioideae seeds, and the embryo can be in direct contact with the seed coat (Fig. 7G) or can be completely surrounded by endosperm and perisperm and not touching the endotesta (Fig. 7H). In some samples the embryo does not fill the embryo cavity and an airspace is present between the embryo and seed coat (Fig. 7I). Embryos of these seeds are mature and considered not touching the seed coat.

### **Alpinioideae seeds in systematic context**

Clades in parentheses correspond to those described in Kress *et al.* 2007. Results are summarized in Tables 2 and 3. No taxa were sampled from the *Alpinia rafflesiana* clade (clade “a”), *Elettariopsis* clade (clade “e”), *Amomum tsao-ko* clade (clade “j”), or *Geocharis* clade (clade “m”) due to lack of material.

**Riedelieae clade** – *Riedelia* Oliv. (1 species analyzed). *Riedelia corallina* (K. Schum) Valeton seeds are ellipsoid, taper slightly at the base and apex, and have a striated surface. They are less than twice as long as they are wide and lack both an external raphe groove and chalazal indentation. The micropylar collar comprises both the endotesta and mesotesta. A hilar rim and micropylar mesotestal proliferation of cells are both absent. The operculum is formed from two layers of the testa with the outermost layer being bulbous cells of the mesotesta (Fig. 4E). The chalazal region has no evident mesotestal modification and no chalazal chamber or chalazal mucro exists. The exotesta is a single layer of palisade cells that are not uniform across the entire seed coat. The mesotesta contains large bulbous cells and the endotesta is a uniform layer of square cells, but whether they are sclereids or parenchyma is unknown (Fig. 6R). An endotestal gap (side?) and CPG cells are present but difficult to see in the material examined. The embryo is straight and does not come in contact with the endotesta.

**Burbidgea** Hook.f. (1 species analyzed). *Burbidgea stenantha* Ridl. seeds are ellipsoid, taper at the base and have a striated surface. They are more than twice as long as they are wide and have no noticeable external raphe groove or chalazal indentation. The micropylar collar is formed from a single layer of cells and the operculum comprises two distinct layers, the outer of which corresponds to the layer of bulbous cells in the mesotesta. A hilar rim and a micropylar proliferation of mesotesta cells are present. The chalazal region is modified into a chalazal mucro (Fig. 5H) and an endotestal gap with CGP cells are present on the side of the seed. The seed coat comprises a uniform palisade exotesta, mesotesta of bulbous cells and a uniform endotesta of square sclereids. The embryo is straight and does not come in contact with the endotesta.

***Pleuranthodium*** (K. Schum.) R.M. Sm. (unknown species analyzed).

*Pleuranthodium* seeds are variously shaped, but the samples investigated were irregularly oblate with a wide equatorial region, and are striated and shiny (Figs. 2M, N). They are less than twice as long as they are wide and contain a chalazal indentation, but lack an external raphe groove. The micropylar collar comprises two distinct layers and the operculum is also two-layered, with an outer layer of bulbous mesotestal cells. A hilar rim and micropylar proliferation and chalazal mucro are all absent. The chalazal region contains a mesotestal proliferation of cells but lacks a chalazal chamber. The exotesta is uniform and palisade and a bulbous layer of mesotesta is present. The endotesta is also uniform and formed from square sclereids (Fig. 6J). An endotestal gap and CPG cells are present at the base of the seed. The embryo is straight and does not touch the inner layer of the endotesta.

***Siamanthus*** K.Larsen & J.Mood (1 species analyzed). *Siamanthus siliquosus*

K.Larsen & J.Mood seeds are ovoid, taper at the base and have a verrucose surface. They are not more than twice as long as they are wide and lack an external raphe groove and chalazal indentation. The micropylar collar is formed from the endotesta only and the operculum comprises two layers. A hilar rim, micropylar and chalazal proliferation of cells, and a chalazal mucro are lacking. The exotesta is made of palisade cells, but is not uniform throughout the seed coat. The mesotesta consists of bulbous cells and the endotesta is a uniform layer of rectangular sclerenchymatous cells. An endotestal gap and CPG cells are present at the base of the seed. The embryo is L-shaped, but does not come in contact with the endotesta.



***Siliquamomum* clade (clade “b,” 1 genus/1 species analyzed).** Seeds of *Siliquamomum tonkinense* Baill. are ellipsoid, taper at the base, and have a striate seed surface (Figs. 2Q, R). They are more than twice as long as they are wide and have a distinct external raphe, but no chalazal indentation. A micropylar collar formed from endotestal and mesotestal cells is present, as is a hilar rim. A micropylar mesotestal proliferation of cells is absent and the operculum is formed from a single layer of cells. No apparent chalazal mesotesta modification exists in this taxon, but a distinct chalazal mucro is present at the base of the seed (Fig. 5I). The exotesta is uniform and palisade and a mesotesta of bulbous cells is lacking. The endotesta is also uniform and comprises a single layer of rectangular sclereids. An endotestal gap is present on the side of the seed and is oval shaped. The embryo is straight, elongated past the endotestal gap, and does not come in contact with the endotesta.

***Alpinia galanga* clade (clade “c,” 1 genus/3 species analyzed).** Seeds of the *Alpinia galanga* clade are oblate, do not taper, and have a shiny striated surface in *A. galanga* (L.) Willd., shiny(?) in *A. conchigera* Griff., and verrucose in *A. nigra* (Gaertn.) Burt. They are less than twice as long as they are wide and contain no external raphe groove (present in *A. nigra*) or chalazal indentation. The micropylar collar is formed from mesotesta and endotesta, and a hilar rim has not been observed. The operculum is two-layered in *A. conchigera* and *A. nigra*, but is made of a single layer in *A. galanga*. The mesotesta is not proliferated in the micropylar region, but is differentiated into a small group of cells in the chalazal region in all three species studied. A chalazal chamber and chalazal mucro are lacking. The endotesta is uniform and palisade in *A. conchigera* and *A. galanga*, but is not uniform or palisade in *A. nigra*. The mesotesta comprises large bodied

cells and the endotesta is uniform in all taxa. The endotesta in *A. nigra* is made of square sclerenchymatous cells (Fig. 6M), while in *A. conchigera* and *A. galanga* they are rectangular in shape (Fig. 6F). Endotestal gaps and CPG cells occur at the base of the seed in all three taxa examined. The embryos of *A. conchigera* and *A. nigra* are basally bulbous (Fig. 7E), but L-shaped in *A. galanga*, and none touch the endotesta.

***Amomum maximum* clade (clade “d,” 1 genus/2 species analyzed)** – Seeds of *Amomum* aff. *glabrum* are ellipsoid and rugose, and those of *A. sericeum* Roxb. are ovoid and striate. Tapering of the seed body is not evident in *A. aff. glabrum*, but is present at the base and apex of *A. sericeum*. Neither species examined is more than twice as long as wide. An external raphe is present in *A. sericeum*, but lacking in *A. aff. glabrum* and neither have evidence of an external chalazal indentation. A micropylar collar is present and formed from endotestal cells only (Fig. 4H). A distinct hilar rim, and micropylar mesotestal proliferation are lacking. The operculum in both taxa is single layered (Fig. 4H). A chalazal modification is absent in *A. aff. glabrum*, but present as a mesotestal proliferation of cells and an *Amomum*-type chalazal chamber in *A. sericeum* (Fig. 5D). A chalazal mucro is lacking in both species. The exotesta is uniform and palisade and the mesotesta is bulbous in *A. sericeum*, but absent in *A. aff. glabrum*. The endotesta is uniform and comprises rectangular shaped sclereids. An endotestal gap and CPG cells are present at the base of the seed in *A. aff. glabrum*, but are located on the side of the seed in *A. sericeum*. The embryo of *A. sericeum* is L-shaped while it is straight in *A. aff. glabrum*; neither embryo touches the endotesta.

***Alpinia fax* clade (clade “f,” 1 genus/1 species analyzed)** – *Alpinia fax* B.L.Burt & R.M.Sm. has ellipsoid seeds that possess a shiny striate seed coat and do not taper.

Seeds are less than twice as long as they are wide and lack an external raphe groove and chalazal indentation. The micropylar collar is formed from endotesta cells only and the operculum has two distinct cell layers. A hilar rim and mesotestal proliferation of cells are both absent (Figs. 3C, D). The chalazal region is characterized by a mesotestal proliferation of cells and an *Alpinia*-type chalazal chamber, but a chalazal mucro was not observed. The exotesta is a uniform layer of palisade cells, and the mesotesta is made of large bulbous cells. The endotesta is also uniform and comprises rectangular sclereids. An endotestal gap and CPG cells are present on the side of the seed, and the embryo is straight and does not touch the seed coat.

***Renealmia* clade (clade “g,” 1 genus/2 species analyzed) – *Renealmia* L.f.** seeds are striate and ellipsoid in *R. lucida* Maas, but ovoid in *R. occidentalis* (Sw.) Sweet. Both seeds taper at the base and are not more than twice as long as they are wide. An external raphe groove and chalazal indentation are absent. The micropylar collar comprises endotesta and mesotesta and in both taxa and a hilar rim is present, though much more pronounced in *R. lucida* (Fig. 4D). The operculum in both taxa is formed from two distinct layers of testa, and a micropylar mesotestal proliferation of cells is absent. In the chalazal region, a proliferation of mesotestal cells and an *Alpinia*-type chamber is evident in *R. lucida*, while *R. occidentalis* contains an *Amomum*-type chamber, and both lack a chalazal mucro. The exotesta is uniform and palisade, and the mesotesta contains bulbous cells (Fig. 6K). The endotesta is uniform and comprises square sclereids with an endotestal gap and CPG cells at the base of the seed. The embryo of *R. lucida* is L-shaped, and is straight in *R. occidentalis*, but neither touches the inner wall of the endotesta.

***Aframomum* clade (clade “h,” 1 genus/2 species analyzed)** – The *Aframomum* K. Schum. clade is characterized by ellipsoid seeds that taper towards the base and apex and have a shiny, striate seed coat (Fig. 2B). Seeds are less than twice as long as they are wide and lack a raphe groove and chalazal indentation. The micropylar collar comprises both mesotesta and endotesta and the operculum is made of two distinct layers. A small hilar rim is evident, as are proliferations in the mesotestal cells in the micropylar region (Figs. 4A, B). The chalazal region is characterized by a symmetrical proliferation of mesotesta cells (Figs. 5A, B), and a chalazal chamber and chalazal mucro are absent. The exotesta comprises a uniform layer of palisade cells, a mesotesta of large bodied cells, and a uniform endotesta with square sclerenchymatous cells (Figs. 6A-C). An endotestal gap and CPG cells are present at the base of the seed. The embryo is straight and elongate and does not touch the endotesta.

***Geostachys* clade (clade “i,” 1 genus/1 species analyzed)** – *Geostachys densiflora* Ridl. has an ovoid seed that tapers at the base and a verrucose seed surface. Seeds are less than twice as long as they are wide and contain no external raphe groove or chalazal indentation. The micropylar collar is formed from a single layer of endotestal cells and the operculum has two distinct layers. A micropylar mesotestal proliferation and a hilar rim are both absent. The chalazal region has a proliferation of mesotestal cells and an *Amomum*-type chalazal chamber, but a chalazal mucro is lacking (Fig. 5F). The exotesta comprises palisade cells that are not uniform throughout the seed coat. The mesotesta is a layer of large bulbous cells and the endotesta is a uniform layer of rectangular sclereids. An endotestal gap and CPG cells are present and originate from

tissues on the side of the seed. The embryo of *G. densiflora* is L-shaped and does not touch the endotesta.

***Alpinia zerumbet* clade (clade “k,” 4 species examined)** – Seeds of the *Alpinia zerumbet* clade are either oblate (*A. stachyodes* Hance), ellipsoid (*A. brevilabris* C.Presl and *A. japonica* (Thunb.) Miq.) or variously shaped (*A. aquatica* (Retz.) Roscoe) with a striate (in *A. aquatica* and *A. japonica*), shiny striate (*A. stachyodes*) or a verrucose surface (*A. brevilabris*) and lack obvious tapering (Figs. 2I, J, O, P). None are more than twice as long and they are wide and may (*A. aquatica*, *A. brevilabris*, *A. stachyodes*; Figs. 2I, J, O, P) or possibly may not (*A. japonica*) have an external raphe groove. An external chalazal indentation is present in *A. aquatica* and *A. brevilabris*, and *A. stachyodes*, but is unknown in *A. japonica*. Micropylar collars are either formed from endotesta and mesotesta (*A. aquatica*, *A. brevilabris*, and *A. stachyodes*) or of endotestal cells only (*A. japonica*). Opercula in all species are two-layered. A micropylar mesotestal proliferation of cells and a hilar rim are both absent for all taxa analyzed. The chalaza has no modifications in *A. aquatica* and *A. brevilabris*, a mesotestal proliferation lacking a chalazal chamber in *A. stachyodes*, and *A. japonica* has a proliferation of cells and an *Alpinia*-type chalazal chamber. No seeds have chalazal mucros. The exotesta is uniform and palisade in *A. stachyodes*; uniform but not palisade in *A. aquatica*; and neither uniform nor palisade in *A. brevilabris* (Fig. 6N) and *A. japonica*. The mesotesta comprises large bulbous cells in all taxa sampled, and the endotesta is a uniform layer of square (*A. aquatica* and *A. brevilabris*) or rectangular (*A. japonica*, and *A. stachyodes*) sclereids. CPG cells and an endotestal gap occur in all taxa and are either at the base (*A.*

*stachyodes*) or at the side (*A. aquatica*, *A. brevilabris*, and *A. japonica*). Embryos in all species examined are L-shaped, and the embryo does not touch the endotesta in all taxa.

***Alpinia zerumbet* subclade (clade “ki,” 3 species examined)** – Seed shape ranges from ellipsoid (*A. haenkei* C.Presl and *A. malaccensis* (Burm.f.) Roscoe) to ovoid (*A. zerumbet* (Pers.) B.L.Burtt & R.M.Sm.) and are either striate (*A. haenkei*) or verrucose (*A. malaccensis* and *A. zerumbet*). None have a tapering seed body. In *A. malaccensis* only, they are twice as long as they are wide. An external raphe is evident in all taxa sampled and extends down two sides of the seed (Figs. 2E, F) and is also found in *A. katsumadai* and *A. oblongifolia*, two taxa not included in recent molecular phylogenies (Kress *et al.*, 2005, 2007). An external chalazal indentation, micropylar mesotestal proliferation of cells and hilar rim are lacking for all taxa sampled. A well-developed micropylar collar formed from endotesta and mesotesta and an operculum comprising two distinct layers are present in all species sampled. A chalazal mucro is not present, but a distinct chalazal modification of the mesotesta is present that forms a column of cells that splits the embryo chamber into two halves at the base (Fig. 5E). An *Alpinia*-type chalazal chamber is present at the top of the columnar mesotesta proliferation of cells in *A. haenkei* and *A. malaccensis*, whereas an *Amomum*-type chamber is present in *A. zerumbet*. The exotesta is palisade in *A. malaccensis* and *A. haenkei* and is not a uniform layer in any taxa sampled. The mesotesta comprises large, bulbous cells and the endotesta is a uniform layer of square cells in *A. haenkei* and *A. zerumbet*, but are rectangular in *A. malaccensis*. An endotestal gap is present at the base of each seed and is associated with CPG cells. All of the seeds have forked embryos that do not touch the endotesta (Figs. 7F, I).

***Plagiostachys* subclade (clade “kii,” 1 genus/2 species analyzed)** – Seeds within the *Plagiostachys* subclade are ovoid and striate (*P. escritorii* Elmer) or ellipsoid and striate (*P. philippinensis* Ridl.) with both forms tapering at the base. Neither taxon has seeds more than twice as long as they are wide and they do not contain an external raphe groove or chalazal indentation. The micropylar collar is formed from endotesta only in *P. philippinensis*, and of both endotesta and mesotesta in *P. escritorii*. The operculum comprises two distinct layers in both taxa and a hilar rim and micropylar mesotestal proliferation of cells are absent. The chalazal region has both a mesotestal proliferation of cells and an *Amomum*-type chalazal chamber, but lacks a chalazal mucro in both species (Fig. 5C). The exotesta of *P. escritorii* is uniform and palisade, but is uniform and not palisade in *P. philippinensis*. Both have a mesotesta of bulbous cells, a uniform endotesta of square sclereids, an endotestal gap, CPG cells located on the side of the seed, and straight embryos that do not contact the endotesta.

***Alpinia carolinensis* clade (clade “l,” 1 genus/2 species analyzed)** – Seeds within the *Alpinia carolinensis* clade (*A. boia* Seem. and *A. carolinensis* Koidz.) are ovoid and striate in *A. boia* (Fig. 2A), but shiny striate in *A. carolinensis*, and taper at the apex (*A. boia*) or base (*A. carolinensis*). Seeds are less than twice as long as they are wide and lack an external raphe groove and chalazal indentation. The micropylar collar comprises endotesta (*A. boia*), or both endotesta and mesotesta (*A. carolinensis*). The operculum is formed from two distinct cell types (Figs. 3A, B). Both a hilar rim and micropylar mesotestal proliferation of cells are absent. The chalazal region lacks a chalazal mucro, but is modified with a proliferation of mesotesta and *Alpinia*-type chalazal chamber in *A. carolinensis*, and contains a proliferation of mesotesta and *Amomum*-type chalazal

chamber in *A. boia*. The exotesta is palisade in both members of the clade, but is uniform in *A. carolinensis* and not uniform and wavy in *A. boia* (Fig. 6D). The mesotesta consists of bulbous cells and the endotesta is a uniform layer of rectangular sclerified cells. An endotestal gap is apparent on the side of the seed and CPG cells are present in both taxa, but difficult to distinguish in *A. carolinensis*. Embryos are elongate and L-shaped and touch the endotesta in *A. boia* (Fig. 7G), but do not in *A. carolinensis*.

***Alpinia eubractea* clade (clade “n,” 2 genera/3 species analyzed)** – Seeds are ellipsoid and verrucose (*Alpinia luteocarpa* Elmer and *A. caerulea* (R.Br.) Benth.) or range from ellipsoid to ovoid and shiny striate (*Vanoverberghia sepulchrei* Merr.). All specimens examined taper at the base and are less than twice as long as they are wide. An external raphe groove and chalazal indentation are only present in *A. caerulea*. The micropylar collar consists of both endotesta and mesotesta, and opercula are formed from two distinct layers (Fig. 4G). A hilar rim, micropylar mesotestal proliferation and chalazal mucro are absent in all taxa surveyed. The chalazal region is not modified in *A. caerulea* and *A. luteocarpa*, but is modified into a mesotestal proliferation of cells in *V. sepulchrei*. The exotesta is uniform and palisade in *A. caerulea* and *V. sepulchrei* (Fig. 6L), and not uniform or palisade in *A. luteocarpa*. All taxa have a bulbous mesotesta, and a uniform endotesta of square sclereids. An endotestal gap and CPG cells are present and on the side of the seed in all taxa analyzed. The embryos range from straight (*V. sepulchrei*) to L-shaped (*A. caerulea* and *A. luteocarpa*) but in neither case do they touch the endotesta (Fig. 7H).

***Hornstedtia* grade (clade “o,” 1 genus/2 species analyzed)** – *Hornstedtia* seeds are ellipsoid and may taper at the apex (*H. conica* Ridl.) or not (*H. leonurus* (J.Koenig)



Retz.). Seed surface is striate and none are more than twice as long as they are wide. An external raphe and chalazal indentation are present. The micropylar collar is formed from endotestal cells only (*H. leonurus*) or of endotestal and mesotestal cells (*H. conica*). The operculum comprises two layers and a hilar rim and micropylar mesotestal proliferation are both absent (Figs. 4I-J). A modified mesotesta in the chalazal region exists as a proliferation of cells in *H. conica*, but is lacking in *H. leonurus*. A chalazal mucro is absent in all taxa. The exotesta ranges from being a non-uniform layer of non-palisade cells in *H. leonurus* to a uniform layer of palisade cells in *H. conica*. The mesotesta is lacking in *H. leonurus*, but is a layer of bulbous cells in *H. conica*. The endotesta of all members is uniform and consists of elongate palisade sclereids (Figs. 6P, Q). An endotestal gap and CPG cells are also present and located at the base of the seed in all taxa examined. The embryos of both taxa are straight and do not contact the endotesta.

***Etlingera* clade (clade “p,” 1 genus/3 species analyzed)** – Seeds range from being ovoid and tapering at the apex [*Etlingera linguiformis* (Roxb.) R.M.Sm. and *E. yunnanensis* (T.L.Wu & S.J.Chen) R.M.Sm.] to ellipsoid seeds that taper at both the base and apex (*E. elatior* (Jack) R.M.Sm.). Seed surfaces are striate in *E. elatior*, and verrucose in *E. linguiformis* and *E. yunnanensis*. No seed is more than twice as long as it is wide and an external raphe and chalazal indentation are present in all specimens sampled (Figs. 2K, L). The micropylar collar is formed from endotestal cells only, and the operculum comprises a single layer in *E. elatior* and *E. yunnanensis* and two layers in *E. linguiformis* (Figs. 3E, F). A hilar rim and micropylar mesotestal proliferation are lacking in all taxa sampled as is any evidence of a modification of chalazal cells, including a chalazal mucro (Fig. 5G). The exotesta is not a uniform layer of cells and

lacks palisade cells. The mesotesta is highly reduced and lacks large bulbous cells. The endotesta is uniform and in all taxa is notably elongate, forming a column of palisade cells that accounts for the majority of the thickness of the seed coat (Fig. 6O). An endotestal gap and CPG cells are present and located at the base of all taxa sampled (Fig. 5G). The embryo of *E. yunnanensis* is straight (Fig. 7A), but those of *E. elatior* and *E. linguiformis* are L-shaped and none touch the inner layer of the endotesta.

***Amomum villosum* clade (clade “q,” 1 genus/4 species analyzed)** – All seeds sampled in the *Amomum villosum* clade are ellipsoid. Seeds may taper at the apex (*A. koenigii* J.F.Gmel. and *A. ochreum* Ridl.), or not at all (*A. lappaceum* Ridl.). They are more than twice as long as wide in *A. lappaceum* and *A. ochreum*, but not so in *A. koenigii*. Seed coats are either striate (*A. ochreum* and *A. koenigii*) or verrucose (*A. lappaceum*). An external raphe groove and chalazal indentation are absent in all members. The micropylar collar is formed from endotesta cells only in *A. koenigii* and *A. lappaceum* while in *A. ochreum* it includes both endotesta and mesotesta. The operculum consists of two distinct layers and a hilar rim was observed in *A. ochreum* only (Fig. 4F). A micropylar mesotestal proliferation of cells is absent, but two types of chalazal modifications have been found. In the chalaza, mesotestal proliferation of cells is present without a chalazal chamber in *A. lappaceum* and *A. koenigii*, and in *A. ochreum* a mesotestal proliferation of cells as well as an *Alpinia*-type chalazal chamber have been observed. A chalazal mucro is not present in any taxa sampled from this clade. The exotesta is palisade in all but *A. koenigii*, and is either uniform (*A. koenigii* and *A. lappaceum*) or not (*A. ochreum*, Fig. 6H). A large bulbous mesotesta is characteristic of this clade and is shared by all members. The endotesta is uniform and with sclereids that

can be square (*A. ochreum*) or rectangular (*A. koenigii* and *A. lappaceum*). An endotestal gap with CPG cells is present and on the side in all taxa sampled. The embryo is straight and does not touch the inner layer of the endotesta.

***Elettaria*** Maton (1 genus/1 species analyzed) – Seeds of *Elettaria cardamomum* (L.) Maton are ellipsoid with no evident tapering and have a rugose surface (Fig. 2D). They are less than twice as long as they are wide and possess a distinct external raphe groove but no chalazal indentation (Figs. 2G, H). A hilar rim and micropylar mesotestal proliferation are absent, and the micropylar collar comprises endotestal cells only. The operculum consists of two distinct layers and the chalazal region has a mesotestal proliferation of cells. The exotesta forms a uniform layer of palisade cells and beneath it is a layer of bulbous mesotesta. The endotesta is also uniform and comprises a single layer of rectangular sclereids. An endotestal gap and CPG cells on the side of the seed are both present in the chalazal region. The embryo is L-shaped and does not contact the inner endotestal wall.

## DISCUSSION

### Systematic significance of seeds of Alpinioideae

Seeds within Alpinioideae are quite variable in terms of their morphology and seed coat anatomy. When viewed in the context of a recently published phylogeny (Kress *et al.*, 2007), we identified several characters of phylogenetic significance using 3D visualizations and digital sections, many of which were not previously documented in studies based on histological sections and surface fractures (e.g., Rodriguez-de la Rosa & Cevallos-Ferriz, 1994; Liao & Wu, 1996, 2000). Characters that most closely correspond

with well-supported clades are operculum layers, seed coat (testa) anatomy, location of the endotestal gap, and structural variation in the chalazal and micropylar regions (Fig. 8). Characters that vary considerably within a clade, and are therefore considered homoplasious, are seed length and surface.

The two tribes Riedelieae and Alpinieae are quite similar in seed morphology and anatomy, and no characters were found to be exclusive of either tribe; however, the number of layers in the operculum was found to differentiate the two tribes with the exception of three taxa. In both tribes the operculum often comprises two layers (one layered opercula being observed only in the *Amomum maximum* clade, *Etlingera elatior*, and *Siliquamomum tonkinense*), with the inner layer derived from the endotesta and the outer layer from the mesotesta. In Alpinieae, the outer layer of the operculum is a torus or donut-shaped mass of cells that are indistinct in SRXTM images (Fig. 4A), but in Riedelieae, the second layer consists of bulbous mesotestal cells that look very similar to the mesotesta in the seed coat (e.g., Fig. 4E, arrow), especially those cells that comprise the micropylar and chalazal mesotestal proliferations seen in some seeds. A few exceptions were found in opercula structure; e.g. the operculum of *Siamanthus siliquosus* resembles Alpinieae in cell structure and the operculum of *Alpinia luteocarpa* and *A. nigra* have bulbous cells more similar to Riedelieae.

The most distinct clades based on seed characters are the *Aframomum* clade, *Alpinia carolinensis* clade, *Alpinia galanga* clade, *Alpinia zerumbet* subclade, *Plagiostachys* subclade, *Amomum maximum* clade, *Etlingera*, and *Renealmia* (Fig. 8). In contrast, the *Alpinia eubractea* clade, *Alpinia zerumbet* clade, *Amomum villosum* clade, and the *Hornstedtia* grade have considerable variation in seed morphology and anatomy

and share no combination of characters that are unique to these groups. The most informative characters were found in micropylar and chalazal mesotestal modifications, the location of the endotestal gap, and the shape of exotestal and endotestal cells. No characters were found to be exclusive of a single clade, but multiple clades in Alpinioideae share features or combinations of features only found within a single clade.

The *Aframomum* clade is identified as a strongly monophyletic lineage within Alpinioideae and has a distinct flask-shaped fruit, a notable synapomorphy for the genus (Harris *et al.*, 2000; Kress *et al.*, 2005). Seeds of the genus are distinctive in having ellipsoid, shiny, striated seeds that are less than twice as long as wide. The presence of both a hilar rim and micropylar mesotesta proliferation are exclusive to *Aframomum* spp. (*Aframomum* clade) and *Burbidgea stenantha* (Riedelieae), but these two genera are easily distinguished from one another by the presence of a second operculum layer of bulbous cells and the absence of a chalazal mucro in Alpinieae (absent and present, respectively, in *Burbidgea*).

The *Alpinia carolinensis* clade includes members with a caducous primary inflorescence bract, tightly clasping tubular bracteoles, and a narrow fleshy labellum adpressed to the stamen (Kress *et al.*, 2005, 2007). It is the only clade to have seeds with a uniform endotesta of rectangular sclereids, an endotestal gap on the sidewall of the seed, and an L-shaped embryo. It shares these features with *Alpinia fax* and *Amomum sericeum*, but *Alpinia fax* differs from *Alpinia boia* in having an *Alpinia*-type chalazal chamber and differs from both *Alpinia boia* and *Alpinia carolinensis* in having a straight embryo. *Amomum sericeum* differs in having a micropylar collar and operculum derived of the endotesta only.

The *Alpinia eubractea* clade is a strongly supported monophyletic clade, but lacks morphological apomorphies (Kress *et al.*, 2007). Seeds show considerable variation within the three taxa sampled and have no combination of characters that were found only in this group. Seeds vary in micropylar and chalazal mesotestal proliferations, endotesta sclereid shape, and endotestal gap location - four characters that were found to be consistent in and informative for identifying other clades.

The *Alpinia galanga* clade was also found to be strongly supported based on molecular data and contains members with distinct branched inflorescences, open bracteoles, a clawed labellum and thin-walled fruits with a hypodermis and large parenchymatous cells in the mesocarp (Liao & Wu, 1996; Kress *et al.*, 2007). Seeds in this clade are uniform in shape (oblate), lack an external raphe groove (except *A. nigra*) and chalazal indentation, and are characterized by the presence of a mesotestal proliferation of cells in the chalazal region and absence of a chalazal chamber. They also have endotestal gaps at the base of the seed and embryos that do not touch the endotesta. Similar features were found in *Alpinia aquatica* and *A. stachyodes*, but these taxa possess an external raphe groove and a distinct chalazal dimple, which are lacking in members of *A. galanga* clade, with the exception of a raphe groove in *A. nigra*.

Members of the *Alpinia zerumbet* clade do not unanimously share any characters or combination of characters that are unique to the clade, but two subclades (*Alpinia zerumbet* subclade and *Plagiostachys* subclade) are both well-supported based on seed characters. The *Alpinia zerumbet* subclade is supported by several characters: the presence of a ‘double’ raphe groove (Figs. 2E, F) that extends down both sides of the seed, a columnar mesotestal proliferation of cells in the chalazal region (Fig. 5E), and a

forked embryo that does not touch the endotesta. This particular combination of morphology was not seen in any other group and was found in all three taxa sampled. It was also found in *A. katsumadai* and *A. oblongifolia*, taxa not included in the molecular analysis of Kress *et al.* (2007). *Alpinia katsumadai* was assigned to section *Alpinia* subsection *Catimbium* by Smith (1990a) based on morphological data, the same section as the species from the *Alpinia zerumbet* subclade we studied here.

The *Plagiostachys* subclade also contains a combination of characters not seen in any other taxon. Seeds sampled have a chalazal mesotestal proliferation of cells, an *Amomum*-type chalazal chamber, square endotestal sclereids, an endotestal gap on the sidewall of the seed, and straight embryos. *Alpinia boia*, *Amomum sericeum* and *Geostachys densiflora* have similar mesotestal proliferations and *Amomum*-type chalazal chambers, but they all differ from *Plagiostachys* spp. in having rectangular endotestal sclereids and L-shaped embryos. *Elettaria cardamomum* is also similar to *Plagiostachys* spp., but differs in endotestal shape (rectangular) and the endotestal gap location (at base).

The *Amomum maximum* clade is either sister to the *Elettariopsis* clade or *Elettariopsis* is nested within the *Amomum maximum* clade based on molecular evidence (Xia *et al.*, 2004; Kress *et al.*, 2007; Droop, 2012). Seeds sampled from the *Amomum maximum* clade *sensu* Kress *et al.* (2007) have a micropylar collar formed from endotestal cells only, an operculum that comprises a single layer of cells, a uniform layer of palisade exotesta, and a uniform layer of rectangular endotestal sclereids. Similar seeds are seen in *Etlingera elatior* and *E. yunnanensis*, but members of this genus differ in their palisade endotestal cells and lack a uniform palisade exotesta.

The *Amomum villosum* clade is characterized by echinate or smooth fruits and has been recognized as a distinct clade in multiple analyses (Xia *et al.*, 2004; Kress *et al.*, 2007; Droop, 2012). Unfortunately members of this clade have a wide range of seed characters and no character, or combination of characters, was found characterize this clade.

Based on molecular data (Kress *et al.*, 2007) *Etlingera* and *Hornstedtia* were recovered as a single clade with *Hornstedtia* forming a basal grade, and seeds surveyed within the two genera are very similar. *Etlingera* seeds have an external chalazal groove, a chalazal indentation, a micropylar collar formed from endotestal cells, no apparent chalazal mesotesta proliferation, a palisade endotesta and an endotesta at the base of the seed. This combination of characters is only seen in *Etlingera* seeds, furthermore the most distinct character, palisade endotestal sclereids, are only found in *Etlingera* and *Hornstedtia*. Although *Etlingera* has characteristic seed morphoanatomy, the two *Hornstedtia* taxa sampled varied greatly and seed structure was not found to be diagnostic within this grade.

*Renealmia* is a well-supported monophyletic genus in Alpinioideae (clade 'g' of Kress *et al.*, 2007). Seeds have a distinct hilar rim, a character shared with *Aframomum* spp. (clade 'h'), *Amomum ochreum* (clade 'q'), *Burbridgea stenantha* (Riedelieae), and *Siliquamomum tonkinense* (clade 'b'), but differ from these taxa by also having a chalazal mesotestal proliferation of cells, an *Alpinia*-type or *Amomum*-type chalazal chamber, an endotestal gap at the base of the seed, and an endotesta of square sclereids. Stellate trichomes on vegetative structures and a basal inflorescence (terminal on leafy shoots in some species) are two characters that, in combination, have been previously identified as



unique to *Renealmia* (Kress *et al.*, 2007) and the combination of seed characters noted above add additional support for this clade.

The identification of many taxa within Alpinieae, in particular those within *Alpinia*, is difficult and few apomorphies exist within the newly revised clades of the tribe (Kress *et al.*, 2005, 2007). Here, we show that a broad sample of seeds within the subfamily demonstrates considerable variation in seed structure within the group, and that distinctive combinations of seed characters occur in many of the Kress *et al.* (2007) recognized clades. This suggests that an expanded analysis of seed structure focusing on chalazal and micropylar seed structure, seed coat characters, and operculum structure will be fruitful in gaining insights into seed evolution within the group.

### **Comparison with other Zingiberales**

The most comprehensive studies on seed coat anatomy in Zingiberales were by Rodriguez-de la Rosa & Cevallos-Ferriz (1994), who examined thirteen species in six of the eight extant families. The seed coat structure we document in Alpinioideae is very different from Musaceae, Strelitziaceae, and Cannaceae, which all have rather uniformly sclerenchymatous layers (Rodriguez-de la Rosa & Cevallos-Ferriz, 1994). Heliconiaceae seed coats have been interpreted as either highly reduced layers in mature seeds, where endocarp forms a protective coat of the seed (Takhtajan, 1985; Simão *et al.*, 2006), or as endotesta (Rodriguez-de la Rosa & Cevallos-Ferriz, 1994; Smith & Benedict, pers. obs.). Further study is needed to clarify if this layer is of integumentary or fruit wall origin. Seed coat structure of Lowiaceae has not been described in the literature. Marantaceae and Costaceae seed coats are similar to Zingiberaceae in being thin and often formed

from a large sclerified endotesta with relatively little mesotesta or exotesta (Rodriguez-de la Rosa & Cevallos-Ferriz, 1994; Benedict, 2012).

Previous papers have documented the presence/absence of chalazal chambers, opercula, and a hilar rim in other families of Zingiberales. Opercula are absent in Cannaceae, and are reduced to absent in Strelitziaceae, Heliconiaceae, and some Zingiberaceae (Zingiberoideae) (Grootjen & Bouman, 1981; Manchester & Kress, 1993; Rodriguez-de la Rosa & Cevallos-Ferriz, 1994). A hilar rim has only been reported in Musaceae (Manchester & Kress, 1993), making it interesting that we find it here for the first time in some Alpinioideae. Developmental studies are needed to determine whether these are homologous structures.

Chalazal chambers have been recognized only within the Musaceae and Costaceae (Grootjen & Bouman, 1981; Manchester & Kress, 1993; Rodriguez-de la Rosa and Cevallos-Ferriz, 1994) and here we demonstrate their presence in Zingiberaceae for the first time. Interestingly this character is not unique to a few taxa in Zingiberaceae, but is present in Zingiberoideae (Zingiberoideae data not shown), the *Alpinia carolinensis* clade, *A. zerumbet* clade and subclades, *Hornstedtia*, and in some members of *Amomum villosum* and *Amomum maximum* clades, though morphologies vary considerably. We further recognize two distinct types of chalazal chamber, a small circular *Alpinia*-type chamber at the base of the seed (Fig. 5E) and a large *Amomum*-type chamber that often connects to a raphe canal that extends from the base of the seed upwards through the mesotesta to the micropyle (Figs. 5C, D, F). The chalazal chambers of Alpinioideae differ in structure from those of Costaceae and Musaceae although all are located in the mesotesta. The chamber in Costaceae is very small and forms a square or rectangle in

longitudinal section, while the chamber in Musaceae is large, sometimes 1/3 or more of the seed and the septum that divides it from the embryo sac is derived from endotesta and inner integument (McGahan, 1961; Manchester & Kress, 1993; Benedict, 2012). Further developmental studies are needed to determine if chalazal structures found in these three families are in fact homologous. In addition, a mesotestal proliferation of cells in the chalazal region has been documented in at least one taxon of each clade studied here with the exception of *Etlingera*. To date, this type of chalazal modification has not been reported for other Zingiberales families, although a broader survey of seed anatomy in the order is needed.

There are many fossil seed taxa that have been attributed to Zingiberales, including *Spirematospermum* Chandler from the Cretaceous-Pliocene of Eurasia and North America, *Striatornata sanantoniensis* Rodriguez-de la Rosa & Cevallos-Ferriz and *Tricostatocarpon silvapinedae* Rodriguez-de la Rosa & Cevallos-Ferriz from the Campanian of Mexico, "*Musa*" *cardiosperma* Jain from the Maastrichtian/Paleocene of India, and *Ensete oregonense* Manchester & Kress from the Eocene of Oregon (Chandler, 1925; Jain, 1961; Manchester & Kress, 1993; Rodriguez-de la Rosa and Cevallos-Ferriz, 1994; Fischer *et al.*, 2009). A clear relationship with any particular family has been difficult to resolve for all of these taxa except *Ensete oregonense*, which is undoubtedly Musaceae (Manchester & Kress, 1993). Although *Spirematospermum* was originally described as a member of Zingiberaceae based on the spirally striated seeds with distinctive palisade exotesta (Koch & Friedrich, 1971; Friis, 1988), the presence of a chalazal chamber in the fossils was later used to ally *Spirematospermum* with Musaceae (Manchester & Kress, 1993; Rodriguez-de la Rosa and Cevallos-Ferriz, 1994; Fischer *et*

al., 2009). Our documentation of a chalazal chamber in Zingiberaceae weakens the current interpretation of *Spirematospermum* as allied with Musaceae, and indicates that the original placement of *Spirematospermum* within or allied to Zingiberaceae based on similarities of seed coat anatomy should be reconsidered. In addition to seed coat anatomy, phytoliths present in *Spirematospermum* are primarily silica sand and occasional globular phytoliths (Koch & Friedrich, 1971; Chen & Smith, 2013), which are similar to phytoliths characterized from Zingiberaceae but are quite different from phytoliths observed in Musaceae (e.g., Chen & Smith, 2013).

## Conclusion

Seeds within the Alpinioideae vary considerably in morphology and anatomy and provide taxonomically useful characters for many recognized clades in the subfamily. Seeds of Riedelieae and Alpinieae are largely distinguishable based on the layering of the operculum. The most informative characters to distinguish clades are micropylar and chalazal mesotestal modifications, the location of the endotestal gap, and the shape of endotestal and exotestal cells. Some clades were not distinguishable by seed characters alone (*Alpinia eubractea* clade, *Alpinia zerumbet* clade, *Amomum villosum*, and the *Hornstedtia* grade), but many were easily recognized for their distinctive seeds (*Aframomum* clade, *Alpinia carolinensis* clade, *Alpinia galanga* clade, *Alpinia zerumbet* subclade, *Plagiostachys* subclade, *Amomum maximum* clade, *Etlintera*, and *Renealmia*). This suggests that an expanded sampling of seeds, in particular those of clades not represented in the current study, will provide more details of natural variation in the group and a deeper understanding of the evolution of Alpinioideae. Additionally, seed

data of extant members is critical to resolving the largely enigmatic fossil record of Zingiberales, which is rich in fruits and seeds.

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Table 1. List of specimens sampled and voucher information. Herbarium abbreviations follow Index Herbariorum (Thiers, continually updated).

Species	Voucher Information
<i>Aframomum daniellii</i> (Hook.f.) K.Schum.	Delft University of Technology, JW van Loon
<i>Aframomum melegueta</i> K.Schum.	US, J. Higgins 44
<i>Alpinia aquatica</i> (Retz.) Roscoe	SING, SNG-57
<i>Alpinia boia</i> Seem.	US, W.J. Kress 79-1071
<i>Alpinia brevilabris</i> C.Presl	US, M. Ramos 30411
<i>Alpinia caerulea</i> (R.Br.) Benth.	SING, JLS-1660
<i>Alpinia carolinensis</i> Koidz.	US, D.H. Lorence 7907
<i>Alpinia conchigera</i> Griff.	SING, GRC-205
<i>Alpinia fax</i> B.L.Burt & R.M.Sm.	US, A.H.M. Jayasuriya 1217
<i>Alpinia galanga</i> (L.) Willd.	US, Shiu Ying Hu 6225
<i>Alpinia haenkei</i> C.Presl	US, A.D.E. Elmer 17662
<i>Alpinia japonica</i> (Thunb.) Miq.	NY, Murataitcitamura 639
<i>Alpinia katsumadai</i> Hayata	Delft University of Technology, JW van Loon
<i>Alpinia luteocarpa</i> Elmer	US, Kress & Kennedy 05-7785
<i>Alpinia malaccensis</i> (Burm.f.) Roscoe	US, C. Saldanha 14771
<i>Alpinia nigra</i> (Gaertn.) Burt	US, W.J. Kress 00-6808
<i>Alpinia oblongifolia</i> Hayata	US, J. Wen 9436
<i>Alpinia stachyodes</i> Hance	US, n.c., 1801
<i>Alpinia zerumbet</i> (Pers.) B.L.Burt & R.M.Sm.	US, Wen 9412
<i>Amomum</i> aff. <i>glabrum</i>	SING, VNM-B-1479
<i>Amomum koenigii</i> J.F.Gmel.	SING, VNM-B-1443
<i>Amomum lappaceum</i> Ridl.	SING, JLS-1667
<i>Amomum ochreum</i> Ridl.	SING, JLS-1670
<i>Amomum sericeum</i> Roxb.	SING, JLS-1273
<i>Burbidgea stenantha</i> Ridl.	SING, GRC-88

<i>Elettaria cardamomum</i> (L.) Maton	Commercially purchased (JCB)
<i>Etlingera elatior</i> (Jack) R.M.Sm.	SING, SNG-56
<i>Etlingera linguiformis</i> (Roxb.) R.M.Sm.	US, W. J. Kress, M. Bordelon, T. Htum 02-7044
<i>Etlingera yunnanensis</i> (T.L.Wu & S.J.Chen) R.M.Sm.	SING, JLS-1717
<i>Geostachys densiflora</i> Ridl.	SING, JLS-1662
<i>Hornstedtia conica</i> Ridl.	SING, SNG-35
<i>Hornstedtia leonurus</i> (J.Koenig) Retz.	SING, SNG-174
<i>Plagiostachys escritorii</i> Elmer	NY, Ramos & Edano 45045
<i>Plagiostachys philippinensis</i> Ridl.	NY, Elmer 16713
<i>Pleuranthodium</i> sp.	NY, Lorence 8840
<i>Renealmia lucida</i> Maas	SING, JLS-1019
<i>Renealmia occidentalis</i> (Sw.) Sweet	MICH, J. Vera Santos 2513
<i>Riedelia corallina</i> (K.Schum.) Valetton	NY, Annable 3639
<i>Siamanthus siliquosus</i> K.Larsen & J.Mood	US, W.J. Kress 99-6358
<i>Siliquamomum tonkinense</i> Baill.	SING, VNM-B-1469
<i>Vanoverberghia sepulchrei</i> Merr.	NY, Ramos & Edano



























